## BRIEF COMMUNICATION

# Specific Expression of the LIM/Homeodomain Protein Lim-1 in Horizontal Cells During Retinogenesis

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***ABSTRACT*** **The LIM/homeodomain transcription factor Lim-1 has been shown to play an essential role in early embryonic patterning during vertebrate development. Here we report the spatial and temporal expression patterns of Lim-1 during retinal development as detected by immunohistochemistry using a specific anti-Lim-1 antibody. By double-immunostaining, we have demonstrated for the first time that Lim-1 is exclusively expressed within the horizontal cell type in the adult retina. In the developing mouse retina, Lim-1 commences its expression in migratory horizontal cell precursors streaming toward the future horizontal cell layer in the ventricular zone. Moreover, its expression during retinogenesis is spatially and temporally coincident with that of the calcium-binding protein calbindin D-28k in horizontal cells. These data together suggest a possible role for Lim-1 in terminal differentiation and maintenance of horizontal cells, and that Lim-1 can serve as a specific molecular marker for the study of horizontal cell specification.** ***Dev Dyn 2000;217:320–325.*** 

**Key words: Lim-1; homeodomain; transcription factor; retinogenesis; horizontal cell**

## INTRODUCTION

The vertebrate retina is a highly specialized sensorineural epithelium consisting of six classes of neurons and one type of glial cell. The neuronal cell bodies are organized into three nuclear layers: the rod and cone photoreceptors reside in the outer nuclear layer; the three types of interneurons–horizontal, bipoar, and amacrine cells, are clustered in the inner nuclear layer; and the output neurons and displaced amacrine cells are located in the ganglion cell layer (Rodieck, 1973; Dowling, 1987). Embryonically, all these cell types are derived from a sheet of seemingly uniform epithelial cells in the optic vesicle, a protrusion of the neural tube. During retinogenesis, although birthdating studies have shown a loose temporal order of generation of the various retinal cell types (Sidman, 1961; Young, 1985), lineage tracing analyses have demonstrated that the local milieu and cell-cell interactions are the primary determinants of retinal cell specification (Turner and Cepko, 1987; Turner et al., 1990).

A critical issue in retinal development is understanding the molecular basis that controls the determination and differentiation of the diverse retinal cell types. A number of transcription factors are found to play a pivotal role in these developmental processes (reviewed in Jean et al., 1998). We sought to identify from the retina LIM/homeodomain transcription factors that might be involved in generating retinal cell type specificity. The LIM/homeodomain proteins are a family of homeodomain-containing transcription factors that also contain two specialized zinc finger motifs named LIM domains. The LIM domain was first recognized in the *C. elegans* Lin-11 (Freyd et al., 1990), rat Isl-1 (Karlsson et al., 1990), and *C. elegans* Mec-3 (Way and Chalfie, 1988). The LIM/homeodomain factors function in multiple developmental processes including pattern formation and cell lineage determination and differentiation. Moreover, most of them have distinct expression patterns in the nervous system and are shown to play an important role in neural development (reviewed in Dawid et al., 1998; Curtis and Heilig, 1998).

Lim-1 is a LIM/homeodomain protein that is expressed in many regions of the nervous system and mesoderm derivatives during development in both *Xenopus* and mouse (Barnes et al., 1994; Fujii et al., 1994; Karavanov et al., 1996; Taira et al., 1992). XLim-1 is also found in a subset of retinal cells in the tadpole but the identity of these cells is unknown (Karavanov et al., 1996). During early embryogenesis, *Lim-1* has been shown to play an essential role in organizer function in

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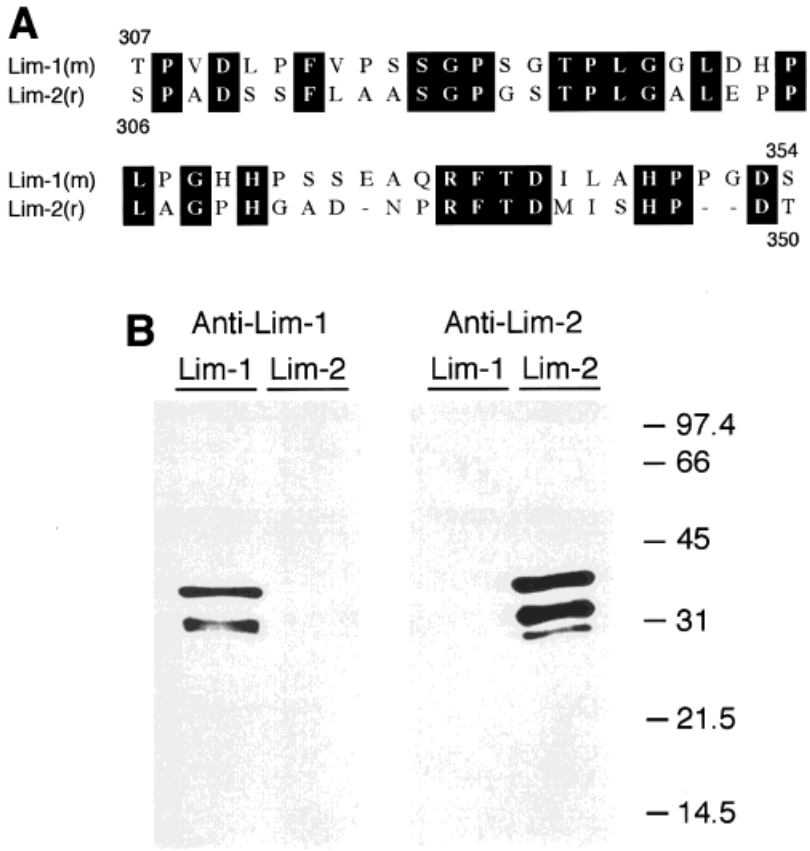


Fig. 1. Specificity of the anti-Lim-1 antibody. **A**: Comparison of amino acid sequences in a diverged region between the mouse (m) Lim-1 and rat (r) Lim-2 that was used for producing the anti-Lim-1 and anti-Lim-2 antibodies. Identical amino acids are outlined. **B**: Immunoblot analysis. Fusion proteins between MBP and the diverged region of Lim-1 and Lim-2 (indicated by Lim-1 and Lim-2 above each lane) were resolved by SDS-PAGE, transferred and immunoreacted with the anti-Lim-1 and anti-Lim-2 antibodies. Protein molecular markers in kilodalton are indicated to the right. Multiple bands are presumed to arise from protein degradation.

both *Xenopus* and mouse (Shawlot and Behringer, 1995; Taira et al., 1994). In the experiments described below, we have demonstrated exclusive expression of Lim-1 in horizontal cells of the retina. Furthermore, Lim-1 is found to be expressed in migratory horizontal cell precursors during mouse retinogenesis.

## RESULTS AND DISCUSSION

### Generation of a Specific Anti-Lim-1 Antibody

To search for novel transcription factors that may be involved in retinal development, we utilized degenerate PCR to amplify from the human retinal cDNA homeobox motifs belonging to the *Lin-11/Mec-3* subclass of LIM/homeodomain genes (Freyd et al., 1990; Way and Chalfie, 1988). Among the clones isolated was a previously reported human homolog of the murine *Lim-1* gene (Dong et al., 1997), suggesting Lim-1 may be expressed in the mammalian retina.

To determine whether Lim-1 is expressed in the retina and what retinal cell type(s) expresses Lim-1, we generated a polyclonal antibody against a mouse Lim-1 region that is most diverged from a closely related protein Lim-2 (Fig. 1A; Tsuchida et al., 1994). In addition, an antibody against the corresponding region in the rat Lim-2 was also produced (Fig. 1A). The mouse Lim-1 and rat Lim-2 share only 46% amino acid identity in this diverged region but they are more than 90% identical in the LIM and homeo domains. To determine the specificity of the anti-Lim-1 antibody, fusion proteins between the Lim-1 and Lim-2 diverged regions and a bacterial maltose-binding protein (MBP) were prepared and reacted with the anti-Lim-1 and anti-Lim-2 antibodies by Western blot assay. As shown in Figure 1B, anti-Lim-1 and anti-Lim-2 recognized only their corresponding fusion proteins and exhibited no cross-reactivity to the other ones, demonstrating anti-Lim-1 is specific. This specificity was confirmed by the inhibition of Lim-1 immunoreactivity in the mouse retina by the Lim-1 but not the Lim-2 fusion proteins (data not shown). Further evidence for the specificity of anti-Lim-1 comes from the fact that immunostaining with anti-Lim-1 gives the same labeling patterns in the mouse brain and otic vesicle as observed in previous studies (data not shown; Barnes et al., 1994; Fujii et al., 1994; Karavanov et al., 1996).

### Localization of Lim-1 in Horizontal Cells of the Adult Retina

To detect Lim-1 expression in the retina, cryosections from adult mouse retinas were immunostained with anti-Lim-1, and simultaneously labeled with the nuclear dye 4′, 6-diamidino-2-phenylindole (DAPI) (Fig. 2A, B). Anti-Lim-1 stained a small population of nuclei lining at the border between the outer plexiform

and inner nuclear layers, a position where horizontal cells are normally situated. Immunostaining mouse whole-mount retinas with anti-Lim-1 revealed that the labeled nuclei were arranged in a regularly spaced mosaic at the level of the outer edge of the inner nuclear layer (Fig. 2G), with an estimated density of 1,100 nuclei/mm$^2$. Since in the mouse retina horizontal cells represent only 0.1–3% of the cells in the inner nuclear layer, corresponding to a density of 100–3,000 cells/mm$^2$ (Young, 1985; Jeon et al., 1998), the relative low density and laminar position of the Lim-1 expressing cells suggest that they are likely to be horizontal cells. To test this possibility, we double-immunolabeled retinal sections with anti-Lim-1, and an anti-calbindin D-28k antibody that strongly stains cell bodies and processes of all rodent horizontal cells and a small portion of amacrine and ganglion cells (Hamano et al., 1990; Uesugi et al., 1992; Peichl and Gonzalez-Soriano, 1994; Oguni et al., 1998; Fig. 2F). Of 140 horizontal cells that scored positive for calbindin D-28k, all were found to express Lim-1 (Fig. 2E, F), suggesting that Lim-1 is expressed in all retinal horizontal cells. A major hurdle in the study of retinal development is the lack of a complete set of cell type-specific molecular markers. The currently available horizontal cell markers including the monoclonal antibody VC1.1 and calbindin D-28K are in particular not exclusively expressed in the horizontal cells (Arimatsu et al., 1987; Alexiades and Cepko, 1997; Fig. 2F). Therefore, the specific expression of Lim-1 in horizontal cells within the retina makes it an ideal horizontal cell marker.

In the macaque retina, anti-Lim-1 also stained horizontal cells present at the outer edge of the inner nuclear layer (Fig. 2C, D), indicating a conserved retinal expression for Lim-1 in the primate. However, this antibody failed to label the chicken retina. This could be due to weak cross-reactivity of this antibody to the chicken antigen as the mouse Lim-1 shares only 75% identity with the chicken Lim-1 in the antigen region, whereas it is 98% identical with the human Lim-1 in the region.

### Temporal and Spatial Expression Pattern of Lim-1 During Retinal Development

During retinogenesis, Lim-1 is weakly expressed in scattered cells of the ventricular zone at E16.5. By E18.5, Lim-1-expressing cells are positioned in a single layer within the ventricular zone of the central retina, presumably in the future perspective horizontal cell layer, even though the outer and inner nuclear layers are not separated by this stage (Fig. 3A, B). In the

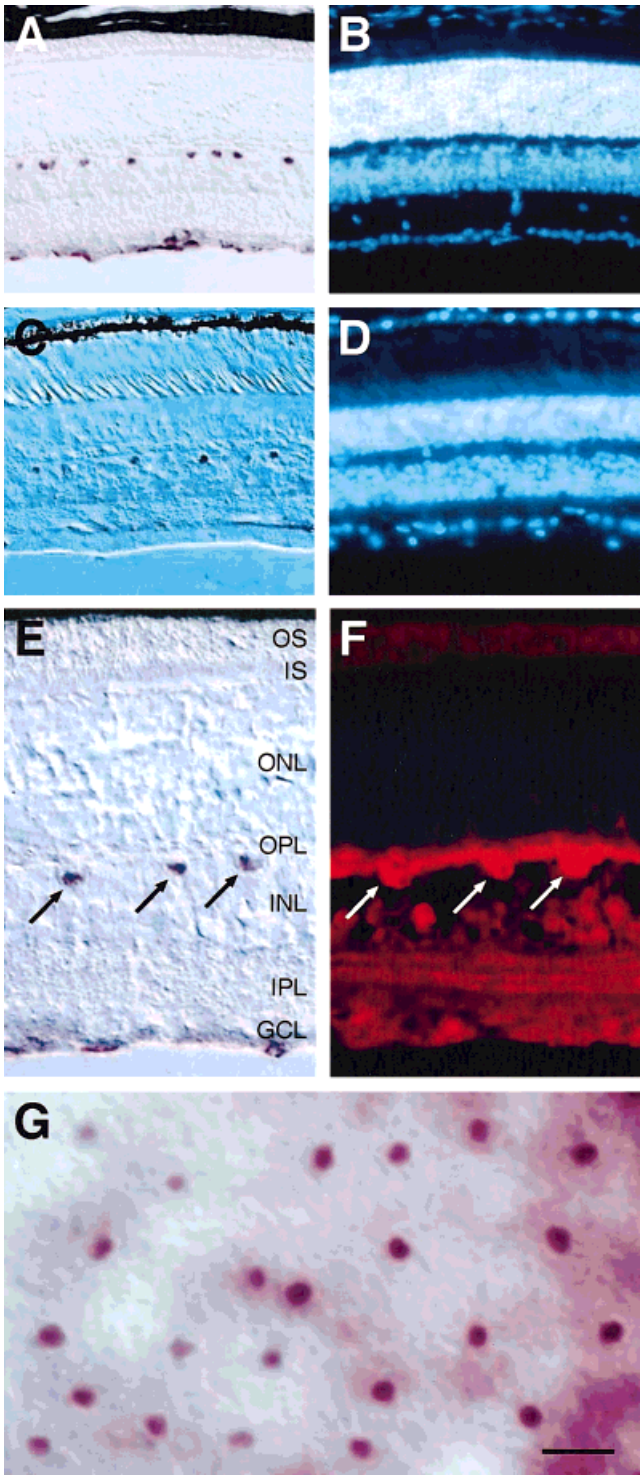


Fig. 2. Identification of Lim-1-expressing cells as horizontal cells in the adult retina. **A–D**: Retinal sections from the mouse (A, B) and macaque (C, D) were double-stained with the anti-Lim-1 antibody (A, C) and DAPI (B, D). Lim-1 is expressed in a single layer of cells lining the outer edge of the inner nuclear layer. **E, F**: Mouse retinal sections were double-immunolabeled with anti-Lim-1 (E) and anti-calbindin D-28k (F) antibodies. Arrows point to colocalized horizontal cells. **G**: Mouse retinal whole-mount was immunostained with the anti-Lim-1 antibody and photographed at the level of the outer edge of the inner nuclear layer. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment. Scale bar = 50 μm in (A–D); 25 μm in (E–G).

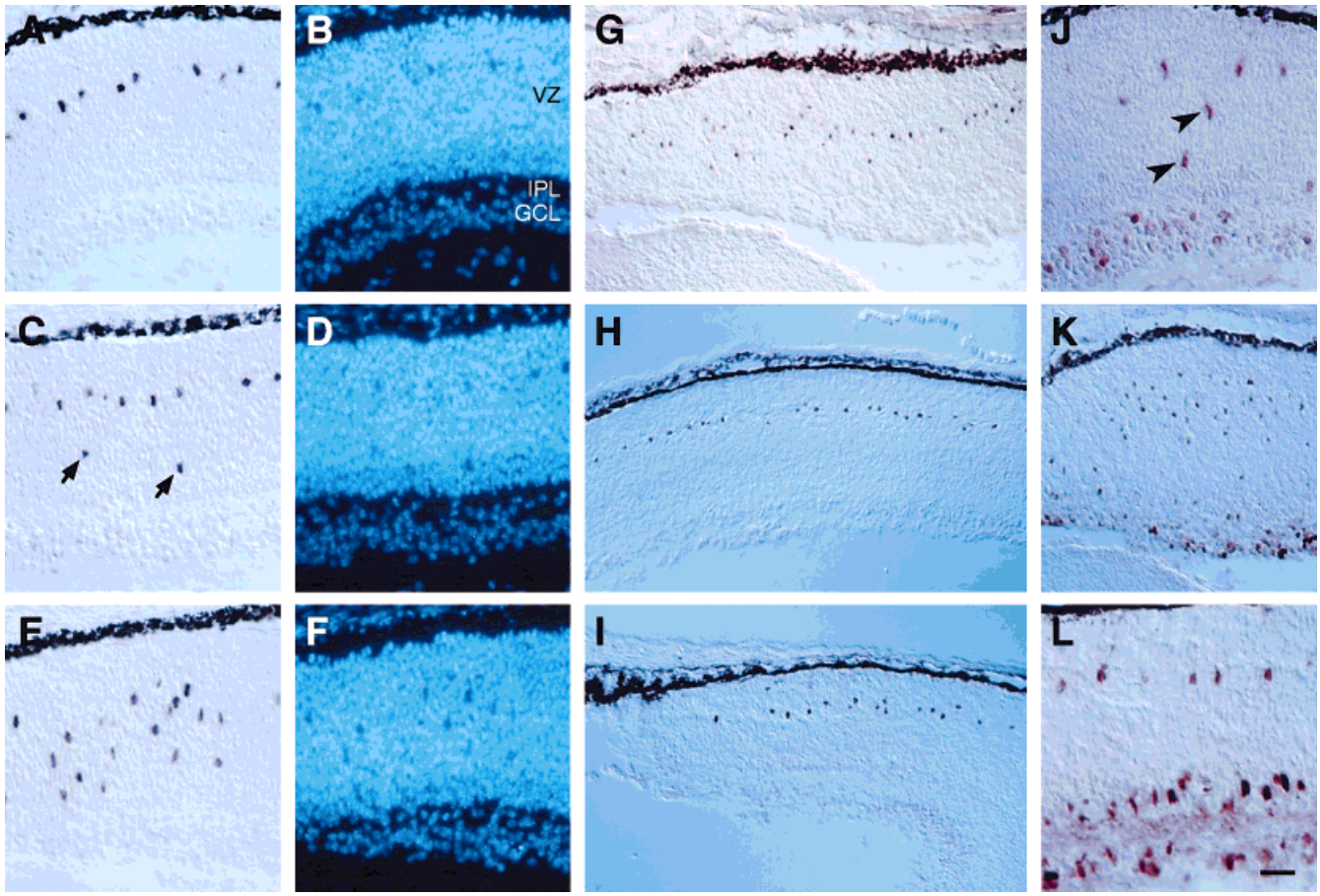


Fig. 3. Comparison of the expression of Lim-1 and calbindin D-28k in developing mouse retinas. **A–F**: E18.5 central (A, B), intermediate (C, D), and peripheral (E, F) retinal sections were double-labeled with the anti-Lim-1 antibody (A, C, E) and DAPI (B, D, F). The Lim-1 expressing cells are arranged in a single layer in the ventricular zone at the center but remain scattered in the periphery. Arrows point to the small number of migrating Lim-1-expressing cells in the intermediate region. **G–I**: Retinal sections from P0 intermediate to peripheral region (G), and P5 central (H), and peripheral (I) regions were immunostained with the anti-Lim-1 antibody. The Lim-1-expressing cells are scattered in the peripheral retina at P0 but positioned in a single layer by P5. The intermediate region is oriented to the right and the peripheral region to the left in (G). **J–L**: Retinal sections from the intermediate region at E18.5 (J), and the peripheral region at P0 (K) and P5 (L) were immunostained with the anti-calbindin D-28k antibody. The expression of calbindin D-28k in horizontal cells displays a similar temporal and spatial pattern as that of Lim-1. Arrowheads indicate migrating horizontal cells that express calbindin D-28k. The sections in J, K, and L were adjacent to those in C, G and I, respectively. GCL, ganglion cell layer; IPL, inner plexiform layer; VZ, ventricular zone. Scale bar = 25 μm in (A–F, J, L); 50 μm in (G–I, K).

intermediate retina, most Lim-1-expressing cells are properly positioned but a small number appear migrating toward the horizontal cell layer (Fig. 3C, D). In the periphery, Lim-1-expressing cells are scattered in the outer two-thirds of the retina (Fig. 3E, F), indicating they have not migrated into their final destination, consistent with the fact that the peripheral retina matures later than the central retina. At P0, Lim-1-expressing cells are located in a single layer in the central and intermediate retina but they remain scattered in the periphery (Fig. 3G). By P5, however, all the Lim-1-expressing cells are confined to a single layer in the ventricular zone of the entire retina (Fig. 3H, I).

In the rat and mouse retina, calbindin D-28k is found in all horizontal cells and a subset of amacrine and ganglion cells (Uesugi et al., 1992; Fig. 2F and 3J–L). Interestingly, the spatiotemporal pattern of Lim-1 expression in the retina closely resembles that of calbindin D-28k expression in horizontal cells. For instance, in the intermediate region of the mouse E18.5 retina, not all horizontal cells that express calbindin D-28k are positioned in a single layer (Fig. 3J); in the peripheral retina, the horizontal cells positive for calbindin D-28k are scattered in the outer two-thirds of the retina by P0, but they are arranged in a single layer in the ventricular zone by P5 (Fig. 3K, L). Therefore, both Lim-1 and calbindin D-28k are expressed in immature horizontal cells and may play a role in their differentiation. The correlation between the expression of Lim-1 and calbindin D-28k also suggests a possibility that Lim-1 may regulate the expression of calbindin D-28k in horizontal cells.

In the mouse all the horizontal cells become postmitotic between E11 and E16 (Young, 1985). Our obser-

vation that the horizontal cell precursors that express Lim-1 are not properly positioned in a single layer in the peripheral retina by P0 suggests a long migration period for the newly produced horizontal cells. Given the usual gestation period of 19.5 days for the C57BL/6J strain that we used in this work, it would take at least 4 days for the newly generated horizontal cells to reach their final destination in the horizontal cell layer. By contrast, the retinal ganglion cells are shown to differentiate and migrate into the ganglion cell layer shortly after their final mitosis (Waid and McLoon, 1995; Xiang, 1998).

In *C. elegans*, genetic analyses of LIM/homeodomain genes have demonstrated a prominent role for them in terminal differentiation of specific neurons. For instance, *mec-3* is required for differentiation of mechanosensory neurons and *lim-6* regulates neurite outgrowth and function of GABAergic motor neurons (Way and Chalfie, 1988; Hobert et al., 1999). The observed spatiotemporal pattern of Lim-1 expression in the retina also suggests a possible role for Lim-1 in terminal differentiation, migration and maintenance of horizontal cells. However, targeted disruption of the *Lim-1* gene in mice causes embryonic lethality at E9.5 due to failure to form the anterior head structures (Shawlot and Behringer, 1995), and is thus unable to shed any light on its role in retinogenesis. It will be interesting to determine whether Lim-1 plays a role in horizontal cell differentiation by tissue-specific knockout and overexpression of Lim-1 in the chick optic vesicle.

## EXPERIMENTAL PROCEDURES

### Amplification and Cloning of LIM-Type Homeobox Motifs

Homeobox motifs belonging to the *Lin-11/Mec-3* subclass of LIM/homeodomain genes were amplified and cloned by degenerate PCR from human retinal cDNA as described in Xiang et al. (1993). The degenerate PCR primers used were as follows: 5′AGTCGAATTC(C/A)GNGGNC(T/C)N(C/A)GNACNACNAT(A/T/C)AA and 5′GTCAGGATCC(A/G)TT(T/C)TG(A/G)AACCANAC(T/C)TG(A/T/G)ATNACNC(G/T)CAT.

### Antibody Production and Western Blotting

DNA segments corresponding to amino acids 307–354 of the mouse Lim-1 and 306-350 of the rat Lim-2 were amplified by PCR (Fig. 1A), and inserted in frame into the pGEMEX (Promega) and pMAL-cR1 (New England Biolabs, Beverly, MA) vectors to express fusion proteins with the bacteriophage T7 gene 10 protein and bacterial maltose-binding protein, respectively. Antibody production, affinity-purification of antibodies, and Western blotting analysis were performed as described previously (Xiang et al., 1993; 1995).

### Immunohistochemistry

Immunostaining and DAPI-labeling of cryosections were performed as previously described (Xiang et al., 1993; 1995). Double immunostaining with the anti-Lim-1 and anti-calbindin D-28k (SWant) antibodies was performed as described in Xiang et al. (1998). Immunostaining of whole-mount mouse retinas was carried out as described in Xiang et al. (1995).

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